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(71) Applicant *(for all designated States except US)*: THE GOVERNEMENT OF THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF THE DEPARTEMENT OF HEALTH AND HUMAN SERVICES [US/US]; Bethesda, MD 20892 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: RAMSEY, William, J. [US/US]; 18 Neerwinder Court, Germantown, MD 20874 (US).

(74) Agents: EINHORN, Gregory, P. et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111 (US).

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(54) Title: REPLICATION DEFICIENT RETROVIRAL VECTOR SYSTEM AND METHODS OF USING

(57) Abstract

The present invention provides a method of producing a replication deficient viral vector encoding a heterologous nucleic acid, said method comprising the following steps: (i) transforming a producer cell with an integrating proviral sequence wherein the integrating proviral sequence comprises a retroviral packaging signal and a sequence encoding the heterologous nucleic acid wherein the proviral sequence is flanked by retroviral long terminal repeats: (ii) infecting the transformed producer cell with a complementing viral vector comprising a complementing genetic sequence wherein the complicating genetic sequence comprises a gene which complements the integrating proviral sequence thereby enabling the producer cell to generate the replication deficient viral factor; and, (iii) culturing the transformed and infected producer cells in a cutlure medium under conditions permissive for the production of the replication deficient viral vectors. It also discloses a cell system and kit for producing a replication deficient viral vector encoding a heterologous nucleic acid and methods of making and using the same.

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REPLICATION DEFICIENT RETROVIRAL VECTOR SYSTEM AND METHODS OF USING

FIELD OF THE INVENTION

The present invention relates to the discovery of a novel means to economically produce replication deficient vectors in large amounts. To date, attempts to produce viral vectors for use in gene therapy report either very low yields or use protocols not adaptable to large scale viral production. These vector systems have focused on a basic strategy: a cell is modified to contain gene(s) able to complement an infecting virus which contains a heterologous gene of interest. This invention, for the first time, encompasses an opposite strategy. The producing cell is transduced with a proviral sequence, containing a heterologous gene of interest and a packaging signal, flanked by LTRs; while the infecting virus contains gene(s) able to complement the proviral sequence. This novel method produces unexpectedly high viral titers in a manner readily adaptable to large scale production techniques.

BACKGROUND OF THE INVENTION

A principal limitation on the use of retroviral vectors for gene therapy *in vivo* is that they are difficult and expensive to make. Specifically, currently available cell systems used to make the replication deficient viruses used in gene therapy result in very low amounts and/or require protocols that cannot be scaled up. These methodologies require significant input of time and costs even to produce enough therapeutic vector to treat a single individual. Addressing this great need in the industry, this invention provides a novel means to produce replication deficient vectors at high titers for use in gene therapy which is readily amenable to large scale production.

The numerous published reports describing means to produce replication deficient retroviral vectors for use in gene therapy report an abysmally low yield, often with titers so low they approximate one to ten viable transducing particles per cell per day. Additionally, many techniques are expensive and cannot be scaled up for bulk production of a "gene therapy" vector. Accordingly, new methods and systems of economically

producing large amounts of replication deficient vectors are clearly needed. The present invention fulfills these and other needs.

Of all the viral vector systems studied to date, retroviral based systems remain the most popular. Retroviral vectors are useful because the genes they transduce are integrated into the genome of target cells, providing long term, stable expression of the heterologous gene. However, retroviral based vector systems have a number of problems. Most importantly, viral titers of retroviral particles produced from packaging cells are low, on the order of 10^6 to 10^7 viral particles/ml.

Other viral based systems have encountered related difficulties. For example, although packaged adenovirus generally has high titers, the viral genome is episomal and transient. In addition, the virus induces an immune response, particularly in the majority of the human population that has been previously infected with this common virus. Similarly, both HSV and vaccinia vectors often provoke an immune response, particularly in those humans that either have been previously exposed to the virus or who have been immunized against the virus.

Accordingly, there is a need for improved eukaryotic viral vectors for diagnostic applications and for delivering heterologous genes to cells *in vitro, ex vivo*, and *in vivo*. This invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The invention provides a method of producing a replication deficient viral vector encoding a heterologous nucleic acid. The method comprises transforming a producer cell with an integrating proviral sequence, where the integrating proviral sequence comprises a retroviral packaging signal and a sequence encoding the heterologous nucleic acid. The proviral sequence is flanked by retroviral long terminal repeats. The proviral sequence, while capable of integration into the host genome, lacks genes which encode certain functions necessary for generation of a replication deficient viral vector, for example, the retroviral gag, pol or env genes. These *trans*-complementing functions are provided by a complementing viral vector. After transformation with the integrating proviral sequence, the producer cell is infected with a complementing viral vector comprising a complementing genetic sequence. The complementing genetic sequence comprises a gene which complements the integrating proviral sequence, thereby enabling the generation of a

replication deficient viral vector in the "complemented" producer cell. As a final step in the method, the transformed and infected producer cell is cultured in a medium under conditions permissive for the production of the replication deficient viral vectors. The method can comprise the additional step of isolating the replication deficient viral vector from the producer cell and the culture medium.

In the method of the invention, the heterologous nucleic acid (initially cloned into the "complementing virus") can encodes a heterologous protein, and the heterologous protein can be a biologically active protein. In various embodiments, the biologically active protein can be a hormone, enzyme, cell receptor, growth inhibitor, antiangiogenic factor, Factor IX, ADA or CFTR.

Alternatively, the heterologous nucleic acid can encode nucleic acids with various functions, *e.g.*, as an antisense nucleic acid or a ribozyme.

In different embodiments, the complementing viral vector can be a vesicular stomatitis virus G or it can be an adenovirus. The complementing gene can encode any protein or nucleic acid necessary to enable the transduced and infected producer cell to generate a replication defective, yet infectious, viral particle, *e..g.*, such as the retroviral genes gag, pol and env, which encode for, *e.g.*, a viral coat protein gene. However, as a complementing gene is any gene that can "rescue" the proviral sequence, it can also be a non-retroviral gene. A complementing gene can also further enable the resultant replication defective virus' infectious potential, *e.g.*, a gene for a vesicular stomatitis virus G coat protein can be included.

The invention also provides a cell system for producing a replication deficient viral vector encoding a heterologous nucleic acid. The cell system comprises a producer cell having an integrated proviral sequence. The integrated proviral sequence comprises a retroviral packaging signal and a sequence encoding the heterologous nucleic acid. The proviral sequence is flanked by retroviral long terminal repeats.

The cell system (comprising the producer cell) also has a complementing viral vector comprising a complementing genetic sequence. The complementing genetic sequence comprises a gene which complements the integrated proviral sequence, thereby enabling the producer cell to generate a replication deficient viral vector. The cell system can further comprise a means to isolate replication deficient viral vectors from producer cells and culture medium. In the cell system, the heterologous nucleic acid encodes a heterologous protein.

The heterologous protein can be a biologically active protein, and the protein can be a hormone, enzyme, cell receptor, growth inhibitor, antiangiogenic factor, Factor IX, ADA or CFTR. The heterologous nucleic acid can encode an antisense nucleic acid or a ribozyme. The complementing viral vector viral vector can be a vesicular stomatitis virus G or an adenovirus. The complementing gene can be a viral coat protein gene.

The invention also provides a kit for producing a replication deficient viral vector encoding a heterologous nucleic acid. The kit comprises two containers. The first container contains a producer cell having an integrated proviral sequence. As with the cell system of the invention, the producer cell's integrated proviral sequence comprises a retroviral packaging signal and a sequence encoding a heterologous nucleic acid. The integrated proviral sequence is flanked by retroviral long terminal repeats.

The kit's second container contains a complementing viral vector. This vector comprises a complementing genetic sequence which complements the integrated proviral sequence to enable the producer cell to generate a replication deficient viral vector. The kit can further comprise a means to isolate replication deficient viral vectors from the producer cells and the culture medium. In the kit, the heterologous nucleic acid can encodes a heterologous protein. The heterologous protein can be a biologically active protein. The biologically active protein can be selected from the group consisting of a hormone, enzyme, cell receptor, growth inhibitor, antiangiogenic factor, Factor IX, ADA or CFTR. The heterologous nucleic acid can encodes an antisense nucleic acid or a ribozyme. In the kit, the complementing viral vector can be a vesicular stomatitis virus G or an adenovirus. The complementing gene can be retroviral gag, pol and env genes, and include other viral genes, *e.g.*, a viral coat protein gene such as a vesicular stomatitis virus G coat protein gene. In an alternative embodiment, in the kit, the first and second containers are combined.

In another embodiment, the kit further comprises an instructional material, wherein the instructional material indicates how to use the producer cell and the viral vector to produce a replication deficient viral vector.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification and the claims.

All publications, patents and patent applications, including GenBank and ATCC library database references, as cited herein, are hereby expressly incorporated by reference for all purposes to the same extent as if fully set forth herein.

DETAILED DESCRIPTION

This invention pertains to the discovery of a novel means to economically produce replication deficient vectors in large amounts for use in, *e.g.*, gene therapy and for delivering heterologous genes to cells *in vitro, ex vivo*, and *in vivo*. The methods and use of the cell systems and kits of the invention result in the production of large amounts of replication defective viruses comprising heterologous genes of interest, *e.g.*, nucleic acid sequences encoding biologically active proteins, ribozymes, antisense sequences, and the like. As discussed below, it is the invention's novel use of proviral vectors, producer cells and complementing viruses that produces these better, surprising results, *i.e.*, a stable producer cell system generating very high titers of replication defective virus.

As noted above, to date, published attempts to produce replication deficient retroviral vectors for use in gene therapy report very low yields, with titers as low as one to ten viable transducing viral particles per cell per day. These vector systems have focused on a basic strategy: a cell is modified to contain gene(s) able to complement an infecting virus which contains a heterologous gene of interest. One possible reason this approach produces very low levels of virus may be that the production of complementing gene products generates a metabolic stress on the cell, resulting in an unstable cell line.

This invention, for the first time, encompasses an opposite strategy. The producing cell is transduced with a proviral sequence containing a heterologous gene of interest (and a packaging signal, both flanked by LTRs); while the infecting virus contains gene(s) able to complement the proviral sequence. This novel method produces surprisingly high viral titers, from about 200 to 500 viral particles per cell per day to as many as 2000 to 10,000 viral particles per day, depending on the exact cell system used, as discussed below. Furthermore, the cell system of the invention is a stable producer cell line which is amenable to economic, large scale production of large amounts of replication defective viral particles.

In this invention, a "chimeric" proviral genome is constructed. It contains two nucleic acid sequences of proviral origin (which can be from two different viruses), a retroviral packaging signal and a pair of retroviral long terminal repeats (LTRs). Inserted

between the LTRs of the proviral sequence is a heterologous gene of interest. This chimeric proviral genome is used to transduce a eukaryotic cell. The proviral genome, enabled by the flanking LTRs, integrates into the cell's genome. The transduced cell (with the stably integrated proviral genome) is characterized as a "producer cell" because, upon further infection with a complementing virus, it is capable of generating a replication defective virus containing the proviral genome.

The provirally transduced cells can be cultured, stored and frozen. In the kit of the invention, they can be stored in a separate container. The containers can be mixed with the complementing virus at the convenience of the operator.

The transduced "producer" cells are then infected (transduced) by complementing virus. This virus is called "complementing" because it contains gene(s) which, when expressed in the producer cell, enables the integrated proviral sequence to be replicated and packaged into a replication defective, but infectious, particle. In one embodiment, the complementing genes include a the gene encoding the vesicular stomatitis virus G coat protein.

In one embodiment, the complementing virus itself is replication defective. Thus, only replication defective particles containing the proviral sequence are produced.

In a variation on this system, different complementing viruses provide complementing proviral packaging components. Thus, in this system, the several complementing viruses work together to direct the production of the final product, the replication defective virus.

These systems are also useful for diagnostic applications, such as excising or "rescuing" known or unknown integrated viral genomes, such as retroviral genomes, from mammalian cells. This "rescue" can help determine the integrated viral genome's relationship to mammalian disease conditions. In addition, these systems are useful for efficiently developing transgenic non-human animals that express a heterologous gene, *in vitro* transduction, and for *in vivo* and *ex vivo* gene therapy.

DEFINITIONS

To facilitate understanding the invention, a number of terms are defined below.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention

belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology* (2d ed. 1994); *The Cambridge Dictionary of Science and Technology* (Walker ed., 1988); and Hale & Marham, *The Harper Collins Dictionary of Biology* (1991). Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "complementing genetic sequence" refers to gene sequence(s) incorporated into a complementing virus which, when expressed in a producer cell (defined below), enables the integrated proviral sequence to be replicated and packaged into a replication defective, but infectious, particle. Thus, the complementing gene(s) are "*trans*-complementing." The complementing gene(s) must be compatible with the proviral signaling (for packaging) sequence (defined below) to enable the producer cell to package an infectious virus. In one embodiment, the *trans*- complementing gene(s) include retroviral gag, pol and env genes. However, as complementing gene(s) include any set of genes that can "rescue" the proviral sequence to generate a replication defective viral particle, they can also comprise non-retroviral genes. For example, in exemplary embodiments, complementing genes include the envelope gene of the vesicular stomatitis virus G and Moloney murine leukemia virus gag-pol protein encoding genes.

The term "complementing viral vector" refers to a chimeric virus which, upon infection of a "producer cell" (comprising an integrated proviral sequence, as defined herein) can provide the necessary components in *trans* to enable the packaging of the proviral sequence into an infectious particle. Several separate complementing viral vectors can be used to either individually or collectively *trans*-complement the integrated proviral sequence. In this manner, the complementing virus(es) are genetically competent to direct production of a proviral containing, replication defective virus particle. This includes rescue of an unknown viral genome with the *trans*-complementing packaging components provided by the complementing viral vector. When a viral genome is rescued, a "replication deficient secondary virus" is produced. The complementing virus can itself be replication deficient. In one exemplary embodiment, the complementing virus comprises an adenovirus genome herpesviruses, poxviruses, alphaviruses.

The term "integrating proviral sequence" refers a proviral genome comprising a heterologous gene of interest and a retroviral packaging signal which is flanked by retroviral LTRs. The proviral sequence, while capable of integration into the host genome, lacks all the genes necessary for generation of a replication deficient viral vector, *e.g.*, gag, pol or env genes. The necessary *trans*-complementing functions are provided by a complementing viral vector. Thus, thee "proviral sequence" is "rescued" with the "complementing virus" of the invention.

The term "retroviral packaging signal" refers to any nucleic acid sequence that can direct the packaging of the viral genome into a viral particle; *e.g.*, the HIV packaging signal sequence designated "PSI." PSI is a retroviral packaging signal (structurally, a stem-loop structure) on the retroviral RNA genome that directs efficient and specific encapsidation of RNA into retroviral particles, as described in detail, below. Accordingly, a "retroviral packaging signal" of the invention includes any RNA sequence, particularly one comprising a secondary structure, such as a stem-loop structure, capable of directing the packaging of the proviral sequence into a viral particle. In a preferred embodiment, the "retroviral packaging signal" is an HIV PSI signal sequence or a functional homologue of that sequence.

The term "replication defective virus" refers to the virus that is produced after transduction by and stable integration of the proviral sequence (as defined herein) followed by infection (transduction) with the complementing viral vector (the chimeric *trans*-complementing virus). The replication defective virus is capable of subsequently transducing a target host cell. It is a packaged virus particle. Thus, the polynucleotide sequence, or genome, of the replication defective virus includes the proviral sequence.

A "producer cell" or a "producer host cell" is a cell with a stably integrated proviral sequence. The producer cell, after infection with the complementing viral vector (the chimeric *trans*-complementing virus), can generate replication defective virus.

A "target cell" or a "target host cell" is a cell that is capable of being infected by the replication defective virus. The replication defective virus can either produces a lytic infection in the target host cell or integrates its genome into the DNA (*i.e.*, chromosome) of the target host cell.

The terms "transforming" and "infecting" incorporate their common usages refers to the ability of a virus to enter a cell via transduction, infection, internalization, transfection or any other means.

The terms "integration" and "capable of being integrated" incorporate their common usages and refer to the ability of the proviral sequence to integrate into the DNA (chromosome) of a producer cell.

The terms "gag, pol and env genes" incorporate their common usages and refer to the corresponding viral or retroviral genes. The retroviral gene "pol" encodes reverse transcriptase (RT). Pol also encodes "integrase," a protein involved in provirus integration into the host genome. The viral gene "gag" encodes "gag proteins" that are viral capsid proteins. The viral gene "env" encodes "envelope proteins," which are viral envelope proteins. These gene are described in further detail, below.

The term "replication deficient" refers to a virus that, without some form of *trans*-complementation, is not capable of producing virus for subsequent infection cycles. Thus, a replication deficient virus can infect a cell but is not independently capable of producing further infectious viral particles of the same type. A replication deficient virus can lack any component necessary to replicate and produce an infectious viral particle. For example, it may lack genes that provide enzymatic functions or structural proteins, *e.g.*, the retroviral gag or pol genes, or an env gene, such as the env gene from a retrovirus or other virus, such as, e.g., the gene encoding the vesicular stomatitis virus G coat protein.

The term "packaging cell" refers to cells which can generate replication-deficient viruses or viral genomes to form virus particles that are capable of infecting another host cell. Such cells include 293 cells, which package adenovirus, and PSI-2 cells or PA317 cells, which package retrovirus. Typically, a nucleic acid encoding a viral genome is introduced into a cell and is then packaged into a viral particle. Some packaging cells contain mutations so that they cannot supply virus genomes to produce infective particles; these packaging cells typically complement a viral genome to produce infective virus particles. Some packaging cells may also provide a viral genome that is packaged to produce infectious viral particles.

A "heterologous polynucleotide sequence" or a "heterologous nucleic acid" is a relative term referring whether a polynucleotide that is related (structurally or functionally) to another polynucleotide, in a manner so that the two polynucleotide sequences are not related

or arranged in the same relationship to each other as in nature. Heterologous polynucleotide sequences include, *e.g.*, a viral versus a mammalian form of a protein, or, *e.g.*, a nucleic acid encoding an antisense versus a sense nucleic acid. Heterologous polynucleotide sequences are considered "exogenous" because they are introduced to the host cell by the hand of man; *e.g*, via transformation, transduction, or infection techniques. However, the heterologous polynucleotide can originate from a foreign source or from the same source. The heterologous polynucleotide sequence can be recombinantly redesigned, *e.g.*, it can be mutated (*e.g.*, site-directed mutagenesis) or linked to another entity as a fusion protein; or, *e.g.*, it can be fragmented (*e.g.*, treated with a restriction enzyme), or, *e.g.*, it can be operably linked to a regulatory element, such as a promoter.

The term "expression cassette" refers to a series of nucleic acid elements that permit transcription of a gene or polynucleotide in a host cell. At a minimum, the expression cassette includes a promoter and a heterologous nucleic acid sequence. Expression cassettes can also include, *e.g.*, transcription termination signals, polyadenylation signals, enhancer elements, and the like. Thus, falling within the definition of "expression cassette" are "expression vectors," "cloning vectors," "viral vectors," and the like, all terms which usually refer to viruses, plasmids or other nucleic acid molecules that are able to transduce and/or replicate in a chosen host cell.

An "antisense" nucleic acid refers to a polynucleotide that is complementary to a target sequence of choice and capable of specifically hybridizing with the target molecules. The term antisense includes a "ribozyme," which is a catalytic RNA molecule that cleaves a target RNA through ribonuclease activity. Antisense nucleic acids hybridize to a target polynucleotide and interfere with the transcription, processing, translation or other activity of the target polynucleotide. An antisense nucleic acid can inhibit DNA replication or DNA transcription by, *e.g*, interfering with the attachment of DNA or RNA polymerase to the promoter by binding to a transcriptional initiation site or a template. It can interfere with processing of mRNA, poly(A) addition to mRNA or translation of mRNA by, for example, binding to regions of the RNA transcript such as the ribosome binding site. It can promote inhibitory mechanisms of the cells, such as promoting RNA degradation via RNase action. The inhibitory polynucleotide can bind to the major groove of the duplex DNA to form a triple helical or "triplex" structure. Methods of inhibition using antisense polynucleotides therefore encompass a number of different approaches to altering expression

of specific genes that operate by different mechanisms (see, *e.g.*, Helene (1990) *Biochim. Biophys. Acta.* 1049: 99-125); as further described, below.

The term "vesicular stomatitis virus G" as used herein refers to a virus of the Order *Mononegavirales*, Family *Rhabdoviridae*, Genus *Vesiculovirus*. Exemplary species include, *e.g.* vesicular stomatitis virus G, Chandipura virus, Cocal virus, Isfahan virus, Maraba virus, Piry virus, vesicular stomatitis Alagoas virus, vesicular stomatitis Indiana virus. See, *e.g.*,U.S. Patent No. 5,789,229.

The term "adenovirus" refers to a virus that is a member of the *Adenoviridae* family, including Genus *Mastadenovirus* and Genus *Aviadenovirus*. Exemplary species include, *e.g*, human adenoviruses strains 1 to 47, with a preferred species as adenovirus type 5, see, *e.g.* Chroboczek (1992) *Virology* 186:280-285.

The terms "retroviral" and "retrovirus" refer a virus that is a member of the *Retroviridae* family. "Lentivirus" is a genus of the *Retroviridae* family, as is "Oncornavirus." Lentiviruses include, e.g., HIV-1 and HIV-2. Oncornaviruses, or "type C viruses" include murine viruses such as Moloney murine leukemia virus (Mo-MuLV), and murine leukemia virus (MuLV).

General Techniques

Nucleic acids for making or using the viral vectors, cell systems, kits and methods of this invention, whether RNA, cDNA, genomic DNA, or hybrids thereof, may be isolated from a variety of sources, genetically engineered, and/or expressed recombinantly. Alternatively, these nucleic acids can be chemically synthesized *in vitro* (see definition of nucleic acids). Techniques for the manipulation of nucleic acids, such as, *e.g.*, subcloning into expression vectors, labeling probes, sequencing DNA, DNA hybridization are described in the scientific and patent literature, see *e.g.*, Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) ("Sambrook"); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997) ("Ausubel"); and, LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, *Part I. Theory and Nucleic Acid Preparation*, Tijssen, ed. Elsevier, N.Y. (1993) ("Tijssen"). Product information from manufacturers of biological reagents and experimental equipment also provide information regarding known biological methods.

Nucleic acids for making or using the invention can also be generated or quantitated using amplification techniques. Suitable amplification methods include, but are not limited to: polymerase chain reaction, PCR (PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, *ed.* Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), *ed.* Innis, Academic Press, Inc., N.Y. (Innis)), ligase chain reaction (LCR) (Wu (1989) *Genomics* 4:560; Landegren (1988) *Science* 241:1077; Barringer (1990) *Gene* 89:117); transcription amplification (Kwoh (1989) *Proc. Natl. Acad. Sci. USA* 86:1173); and, self-sustained sequence replication (Guatelli (1990) *Proc. Natl. Acad. Sci. USA* 87:1874); Q Beta replicase amplification (Smith (1997) *J. Clin. Microbiol.* 35:1477-1491, automated Q-beta replicase amplification assay; Burg (1996) *Mol. Cell. Probes* 10:257-271) and other RNA polymerase mediated techniques (*e.g.*, NASBA, Cangene, Mississauga, Ontario).

Viruses, vectors, nucleic acids and proteins (*e.g.*, the polypeptide encoded by the heterologous nucleic acid expressed *in vivo*) are detected and quantified by any of a number of general means well known to those of skill in the art. These include, *e.g.*, analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, Southern analysis, Northern analysis, Dot-blot analysis, gel electrophoresis (*e.g.*, SDS-PAGE), RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

Integrating Proviral Sequences

The cell system and methods of the invention comprise transforming a producer cell with an integrating proviral sequence, where the integrating proviral sequence comprises a retroviral packaging signal and a sequence encoding the heterologous nucleic acid. Any retrovirus, portions of other viruses, can be used as the source for the required integrating provirus sequences, *e.g.*, lentiviruses, Oncornaviruses, papilloma viruses, adenoviruses, and alphaviruses. Preferred proviral sequences include those derived from the family *Retroviridae*, see, *e.g.*, White & Fenner, *Medical Virology* (4th ed., 1994); Liljestrom (1991)

Biotechnology 9:1356-1361. A proviral sequence can be derived from amphotropic murine leukemia virus-related "type C" viruses that have been used extensively for gene transfer, *e.g.*, Moloney murine leukemia virus (Mo-MuLV) (for the nucleotide sequence of Mo-MuLV, see, *e.g.*, Shinnick (1981) *Nature* 293:43-548). Other retroviruses as a source of proviral sequences include, *e.g.*, monkey, cat, bird, and human retroviruses. Other proviral sequences include those derived from the family *Togaviridae*, more preferably alphaviruses, *e.g.*, Semliki Forest Virus (SFV); Sindbis Virus (see, *e.g.*, White & Fenner, *supra*); alphavirus, *e.g.*, Semliki Forest virus (SFV) or Sindbis virus. The alphavirus sequences typically required for production of an secondary virus include genes that encode packaging components, *e.g.*, C, p62, and E1. This proviral genome typically includes the genes encoding replicase (NSP1-4) operably linked to a promoter, a genomic promoter, and a heterologous gene operably linked to a subgenomic promoter.

Retroviral Packaging Signals

The proviral genome also includes any packaging signal required for packaging of the proviral genome. For example, in one embodiment, the HIV retroviral packaging signal, designated "PSI," allows packaging of the retroviral genome into infectious viral particles. Encapsidation of the HIV-1 genomic RNA is mediated by specific interactions between this RNA packaging signal and the Gag protein. During maturation of the virion, the Gag protein is processed into smaller fragments, including the nucleocapsid (NC) domain which remains associated with the viral genomic RNA, specifically, to the HIV-1 PSI stem-loop structure. See, *e.g.*, Damgaard (1998) *Nucleic Acids Res.* 26:3667-3676; Clever (1995) *J. Virol.* 69:2101-2109.

Retroviral Long Terminal Repeats

The proviral genome also includes long terminal repeat sequences, *e.g.*, sequences from the 5' and 3' ends of the retroviral genome. The LTR sequences flank the heterologous gene and the packaging signal. The LTR can also include a retroviral promoter which can contribute to the regulation of proviral genome transcription after provirus integration into the producer cell, or, after packaging into the replication deficient viral particle. LTR sequences are also involved in proviral production and integration. The LTR of the invention can be derived from any retroviral source, see, *e.g.*, Parent (1998) *Arch. Virol.* 143:1077-1092; Damond (1998) *J. Clin. Microbiol.* 36:809-811; Voevodin (1997) *Virology* 238:212-220.

Heterologous Nucleic Acids

In one embodiment of the invention, the proviral sequence comprises a heterologous nucleic acid. The proviral sequence is incorporated into a replication defective virus after its "rescue" by the complementing virus. After its isolation from the producer cell and the culture medium, the replication defective virus is used to infect a target cell, for, *e.g.*, gene therapy. Typically, expression of the heterologous gene in the target cell will confer a therapeutic benefit to that cell.

The heterologous gene, in addition to being useful for, *e.g.*, gene therapy (*e.g.*, a nucleic acid encoding a biologically active protein, a ribozyme, an antisense message), can also be useful for selection after viral infection (*e.g.*, antibiotic resistance) or identification of transduced cells (*e.g.*, a fluorescent green protein, or an epitope tag). The coding sequence can also be operatively linked to "control elements" or "transcriptional regulatory sequences." These systems can be manipulated to vary in their strength and specificities. They can include non-translated sequences, *e.g.*, enhancers, promoters, and 3' untranslated regions which interact with host cellular proteins to carry out transcription and translation. Depending on the particular vector system, any number of suitable transcription and translation elements, including constitutive, inducible or cell specific promoters, may be used.

In mammalian systems, especially for vectors for expression in humans *in vivo*, promoters from the mammalian genes or from mammalian viruses are most appropriate. Examples of constitutive promoters include elements from mammalian viruses such as, *e.g.*, cytomegalovirus (CMV), rous sarcoma virus, simian virus 40, Moloney murine leukemia virus, see, *e.g.*, Bui (1997) *Hum. Gene Ther.* 8:2173-2182; Qin (1997) *Hum. Gene Ther.* 8:2019-2029. Example of inducible promoters include, *e.g.*, tetracycline- regulated promoters, see, *e.g.*, Massie (1998) *J. Virol.* 72:2289-2296; glucocorticoid- responsive promoter, see, *e.g.*, Narumi (1998) *Blood* 92:822-833. An examples of cell- specific promoters include, *e.g*, the nerve specific promoter of the peripheral myelin protein 22 gene, see, *e.g.*, Nelis (1998) *J. Med. Genet.* 35:590-593.

Transcriptional control elements can also include enhancer sequence elements. These elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. The retroviral U3 region enhancer, the SV40

early gene enhancer, polyoma virus enhancer, and human or murine cytomegalovirus enhancer are all suitable for inclusion in an expression cassette.

The promoters, enhancers, or other transcriptional regulatory elements used in this invention can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries, or prepared by synthetic or recombinant methods, as described herein.

Polyadenylation sequences are also commonly used. Termination and polyadenylation signals that are suitable for the present invention include those derived from, *e.g.*, the retrovirus LTR sequences. Other suitable sequences include polyadenylation and termination sequences derived from SV40, or a partial genomic copy of a gene already resident on the complementing viral vector.

The expression systems can also optionally contain a variety of other components; including, *e.g.*, sequences permitting replication of the cassette both *in vitro* and *in vivo, e.g.*, eukaryotes or prokaryotes, or a combination thereof, (*e.g.*, shuttle vectors) and selection markers for the selected expression system, *e.g.*, prokaryotic or eukaryotic systems.

Transducing Cells with Proviral Sequence

Many cell types are suitable for transduction by the proviral sequence for the creation of a producing cell line. Use of autologous producer cells are advantageous because they help alleviate any possible immune response to the replication deficient viral particle if it is used as a gene therapy vector. Examples include, *e.g.*, HeLa cells and NIH 3T3 cells. In addition, producer cells can be chosen of the basis of a haplotype which matches the individual who is to receive the replication defective virus particles (generated after *trans*-complementation of integrated proviral sequence by the complementing virus).

Suitable methods for transduction are known to those skilled in the art. Cells are infected after titering virus particle-containing cell supernatants according to standard methods, such as, *e.g.*, Sambrook; Bunnell & Morgan, *Retrovirus-Mediated Gene Transfer*, in *Viral Genome Methods*, pp. 3-23 (Adolph ed., 1996). In one embodiment, cells are cultured to 60-80% confluency in appropriate media. Typically, as was used with D54 and A375 cells in experiments described in the Example section, Dulbecco's Minimal Essential Media (DMEM) with 10% bovine calf serum is used. Cells are transduced in a minimal volume of growth media (approximately 1 ml per 50 square centimeters (cm^2) of culture surface) for about 4 to 12 hours. The optimal multiplicity of infection varies dependent upon the cell line. Typically, the general formula: plaque forming units (pfu) required for 100%

transduction with a marker virus is one to thirty effective transducing units (EFU) per cell for maximal production. There is significant leeway on the lower end (any number greater than one should work in theory) but this will give maximal virus production if the cell line is able to support production. It is possible to give too much virus, *e.g.*, the toxicity of the adenovirus kills the cells before significant retrovirus production occurs, *e.g.*, 100 EFU per cell will kill all cells in the dish, with no retrovirus recovered.

After establishment of a stable cell line, the integrated proviral sequences can be analyzed by any technique, *e.g*, with cDNA synthesis using the integrated proviral genome as template. Alternatively, the integrated genome can be amplified, *e.g.*, with PCR (see above for alternative amplification methodologies), followed by sequencing.

In general, viral genomes can be characterized by any of a number of means well known to those of skill in the art. These methods include the detection of specific polynucleotide by well known methods such as Southern analysis, northern analysis, dot blot analysis, gel electrophoresis, PCR, and RNase protection assays.

Complementing Vectors

In the cell systems and methods of the invention, after transformation with the integrating proviral sequence, the producer cell is infected with a complementing viral vector comprising a complementing genetic sequence. The "complementing viral vector" of the invention contains a chimeric genome, where the genome typically comprises viral nucleic acid sequences from two different types of viruses. First, the genome includes nucleic acid sequences from a "primary" virus to enable the packaging of the complementing virus. The primary viral genome can itself be replication deficient, needing, *e.g.*, complementation of genes, such as for coat proteins, to enable infection of the producer cells of the invention. In alternative embodiments, the complementing viral genome includes elements from vesicular stomatitis virus G or adenovirus.

Second, the chimeric "complementing virus" genome includes "complementing genes," *i.e.*, nucleic acid sequences which encode packaging components capable of *trans*-complementing the integrated proviral sequence to generate an infectious, yet replication-defective, viral particle (described further, below).

Third, the "complementing virus" optionally includes a secondary viral genome that can either integrate into a target host cell after infection or produce an lytic infection (see,

e.g. Fassati *et al.*, *Retroviral Vectors*, in *Molecular and Cell Biology of Human Gene Therapeutics* pp. 1-19 (Dickson ed., 1995)).

Any primary virus can be used as an initial source for the complementing viral genome. Preferably, the primary virus has a broad host range. Primary viruses can include, *e.g.*, vesicular stomatitis virus, adenovirus, vaccinia virus, Epstein Barr virus (see, *e.g.*, Grignani (1998) *Cancer Res.* 58:14-19), and herpes simplex virus (HSV).

Typically, the complementing virus is itself replication deficient (*i.e.*, it cannot produce additional virus) and lacks a gene required for replication or packaging itself (independent of its ability to "rescue" the integrated proviral sequence). For example, is an adenoviral vector is used, it usually has the E1A gene deleted from its genome. This gene, essential for viral replication, is complemented by initially the packaging the virus in cell line 293. Thus, a replication deficient complementary viral genome is packaged after introduction of the viral genome into 293 cells. Other genes that are commonly deleted in adenovirus vectors are E1B and E3.

The complementing virus is packaged by any means suitable in the art (see, *e.g.*, Sambrook, *supra*, Ausubel, *supra*). If the complementing virus itself is replication defective, it will need its own *trans*-complementing cell line. A commonly used *trans*-complementing cell line for packaging retrovirus is, *e.g.*, PA317 (Miller (1986) *Mol. Cell. Biol.* 6: 2895-2902). A commonly used *trans*-complementing cell line for packaging adenovirus is the transformed primary human embryonal kidney cell line 293 (Roizman (1996) *Proc. Natl. Acad Sci USA* 93: 11307-11312).

The complementing viruses are collected and used to infect a producer cell by any standard methodology (see, *e.g.*, Sambrook, *supra*, Ausubel, *supra*).

Vesicular Stomatitis Virus G (VSV-G) Vectors

In one embodiment of the invention, the complementing virus comprises elements of vesicular stomatitis virus (VSV), such as VSV-G. VSV vectors are well-known to be suitable means to transduce cells, such as the producer cells of the invention. Further, the complementing viral genome can contain coding sequence for viral elements that contribute the infectivity of the replication defective virus, such as vesicular stomatitis virus coat protein gene. See, *e.g.*, U.S. Patent No. 5,512,421; U.S. Patent No. 5,739,018.

Adenovirus Vectors

In one embodiment of the invention, the complementing virus comprises elements of adenoviruses from the family *Adenoviridae* (White & Fenner, *Medical Virology* (4th ed., 1994)). Adenoviral vectors are well-known to be suitable means to transduce cells, such as the producer cells of the invention. Further, the complementing viral genome can contain coding sequence for viral elements that contribute the infectivity of the replication defective virus, such as adenoviral coat protein (similar to use of vesicular stomatitis virus coat protein gene, as described above).

In one embodiment, the primary virus is derived from the strain adenovirus 5 (for the sequence of ad 5, see Chroboczek (1992) *Virology* 186:280-285). The use of adenoviral vectors *in vitro*, *in vivo*, and for gene therapy, is well described in the patent and scientific literature, *e.g.*, see, Hermens (1997) *J. Neurosci. Methods*.71:85-98; Zeiger (1996) *Surgery* 120:921-925; Cannon (1996) *Cardiovasc Res*. 32:962-972; Huang (1996) *Gene Ther.* 3:980-987; Zepeda (1996) *Gene Ther*. 3:973-979; Yang (1996) *Hum. Mol. Genet.* 5:1703-1712; Caruso (1996) *Proc. Natl. Acad. Sci. USA* 93:11302-11306; Rothmann (1996) *Gene Ther*. 3:919-926; Haecker (1996) *Hum. Gene Ther*. 7:1907-1914; to name just a few. The use of adenoviral vectors is also described in, *e.g.*, U.S. Patent No. 5,792,453; U.S. Patent No. 5,731,172; U.S. Patent No. 5,670,488; U.S. Patent No. 5,585,362. Adenovirus type 5 and adenovirus type 2 genomes are also described, *e.g.*, by Chroboczek (1992) *Virology* 186:280-285.

Complementing Genes

In the cell systems and methods of the invention, complementing genetic sequences are gene sequence(s) incorporated into a complementing virus which, when expressed in a cell, enables the integrated proviral sequence to be replicated and packaged into a replication defective, but infectious, particle. These complementing gene(s) should be compatible with the proviral signaling/packaging sequence to generate a viral particle containing the proviral genome. While *trans*- complementing genes typically include one or all of the retroviral gag, pol and env genes, they can comprises genes from any source, including non-retroviral genomes. The "complementing genetic sequence" can encode any "packaging signal" or "packaging component" required for assembling a proviral genome. The "complementing genetic sequence" can also contain additional viral sequence, for, *e.g.*,

contributing to the infectivity of the replication defective virus, such as, *e.g.*, vesicular stomatitis G coat protein.

In one embodiment, the complementing genes comprise the retroviral gag, pol and env genes, which are well described by the scientific and patent literature. The retroviral gene "pol" encodes reverse transcriptase (RT). RT has several activities, including reverse transcription and RNase H activity, that are necessary for transcribing the retroviral RNA genome into provirus DNA. Pol also encodes "integrase," a protein involved in provirus integration into the host genome. RT and integrase proteins are packaged into the infective replication defective virion particle along with the proviral RNA genome. The retroviral gene "gag" encodes "gag proteins" that are viral capsid proteins. The retroviral gene env encodes "envelope proteins," which are viral envelope proteins.

Specific Targeting of Target Cells Using Complementing Genes

Replication deficient viruses of the invention can be constructed that are selectively targeted to target host cells and tissues. Examples of targeted tissues include skin, blood, and airway epithelium. Selective targeting is accomplished, *e.g.*, by the choice of complementing gene. For example, a replication defective virus can be targeted by incorporating various retroviral env genes, each of which acts as a cell-specific ligand for a receptor on the target cell. Thus, manipulating or pseudotyping the complementing env gene can alter the tropism of the virus particle. For example, amphotropic murine leukemia virus (ampho MuLV) env shows few trophic specificities, whereas gibbon ape leukemia virus (GALV) env has much higher transduction efficiencies for mature T-lymphocytes.

Replication deficient viruses of the invention can also be constructed to preferentially target "stem cells," the self-renewing population of cells which serve as the source of the terminally differentiated cells. Amphotropic env can be used to target CD34, an antigen present on hematopoietic stem cells (Bunnell (1997) *Blood* 89:1987-1995). Such targeting would provide an efficient means of gene therapy, since the stem cells containing the heterologous gene of choice would provide populations of differentiated cells that also contained this gene. In addition, if the heterologous gene conferred a biologically selectable advantage, *e.g.*, better growth or resistance to toxins, repopulation of the target organ with genetically altered cells could proceed more completely and more rapidly.

Infecting Cells with Complementing Vectors

A complementing virus is typically introduced into producer cells by standard methodology, *e.g.*, transfection, electroporation and the like (as noted above, producer cell lines can be, *e.g.*, HeLa cells, NIH 3T3 cells, and the like). Suitable methods for infection are known to those skilled in the art. Cells are infected after titering complementing virus-containing solutions according to standard methods. See, *e.g.*, Sambrook, *supra*, Ausubel, *supra*, Bunnell & Morgan, *supra*.

Typically, complementing viruses infect proviral genome-containing producer cells at multiplicities of between about 10 to about 30 adenovirus plaque forming units (PFU)/ cell/ virus. See, *e.g.*, Graham (1992) *Biotechnology* 20:363-390. Additional examples of means to introduce trans-complementing virus include, *e.g*, use of Semliki Forest virus (SFV) expression to mediate cytoplasmic synthesis of retrovirus vector RNA, as in Wahlfors (1997) *Hum. Gene Ther.* 8:2031-2041; or, using alphavirus RNA introduced by electroporation rather than infection to produce the trans-complementing functions (see, *e.g.*,Wahlfors (1997) *supra*; Li (1998) *Proc. Natl. Acad. Sci. USA* 95:3650-3654. Alternatively, transfection with plasmids can be used to introduce plasmids which provide the *trans*-complementing functions (see, *e.g.*, Kinsella (1996) *Hum. Gene Ther.* 7:1405-1413).

As discussed above, if the replication defective virus particle is to be used in gene therapy, it is desirable to use a producer cell line (containing the proviral sequence with the heterologous gene of interest) that has the same or similar MHC haplotype as the intended recipient. Alternatively, if the producer cell line is not matched to the haplotype of the gene therapy recipient, they can be irradiated before introduction of the complementing virus.

Means to Isolate Replication Deficient Viral Vectors

After infection with the complementing virus, producer cells are incubated under standard cell culture conditions to allow packaging of the proviral genome and budding of replication deficient viral particles into the cell supernatant. The cells and/or cell culture media (supernatant) are collected after a suitable amount of time, *e.g.*, after 24 hours, depending on the exact culture conditions, and the like. These samples are then frozen or used immediately. The viruses can be harvested and isolated by standard methodologies well described in the patent and scientific literature, see, *e.g.*, Sambrook, *supra*, Ausubel, *supra*, Bunnell & Morgan, *supra*.

The biological activity (*e.g.*, range of infectivity) of these viral particles can be tested by any means, *e.g.*, infecting any suitable cell type and examining for intracellular presence of the genome of the replication deficient virus, expression of the heterologous gene, and/or integration of the proviral sequence. The presence of replication deficient viral genomes can also be directly analyzed, as discussed above.

Use of Replication Deficient Viral Vectors

The replication deficient viruses confer a therapeutic benefit to the target host cell by delivering the heterologous gene of interest. The replication deficient viruses can be designed to producing a lytic infection or an integrative event. Generally, integrative replication deficient viruses are used for long term treatment, and lytic viruses are used for transient or anti-proliferative treatments. Integrative viruses can be used to, *e.g.*, provide functional genes to cells that contain defective copies. Alternatively, they can also be used in functional or anti-proliferative therapeutics by providing antisense or ribozyme nucleic acids.

Gene Therapy

The replication deficient viruses of the invention are used for mammalian gene therapy, preferably for human gene therapy. Gene therapy provides methods for combating infectious diseases such as HIV infection, as well as non-infectious diseases and conditions, such as cancer and birth defects (see generally Anderson (1992) *Science* 256:808-813; Yu (1994) *Gene Ther.* 1:13-26). For example, suitable therapeutics can target RNAs (*e.g.*, using ribozymes or antisense RNA), proteins (RNA decoys, transdominant proteins, intracellular single chain antibodies, soluble CD4), infectable cells (suicide genes), or the immune system (*in vivo* immunization). Gene therapy can be used to transduce cells with either an *ex vivo* or an *in vivo* procedure.

Biologically Active Proteins

When the replication deficient viruses of the invention is used in gene therapy, typically the heterologous protein inserted into the proviral genome is a biologically active protein. Example of useful biologically active proteins include, *e.g.*, hormones, enzymes, cell receptors, growth inhibitors, antiangiogenic factors; specific examples being, *e.g.*, Factor IX, ADA or CFTR.

Antisense Nucleic Acids

In one embodiment of the invention, the heterologous gene of the proviral genome encodes an antisense sequence. Antisense sequences are capable of inhibiting the transcription of genes or the transport or splicing of messages by targeting genomic DNA or messenger RNA respectively. The transcription or function of targeted nucleic acid can be inhibited, for example, by hybridization and/or cleavage. Strategies for designing antisense oligonucleotides are well described in the scientific and patent literature.

Ribozymes

The invention also provides for heterologous genes which encode ribozymes capable of targeting mRNA. Ribozymes act by binding to a target RNA through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the RNA that cleaves the target RNA. Thus, the ribozyme recognizes and binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cleave and inactivate the target RNA. Strategies for designing ribozymes are well described in the scientific and patent literature, see, *e.g.*, Rossi (1992) *Aids Research and Human Retroviruses* 8:183; Hampel (1990) *Nuc. Acids Res.* 18:299; Perrotta (1992) *Biochemistry* 31:16; Guerrier-Takada (1983) *Cell* 35:849; Cech, U.S. Pat. No. 4,987,071.

"Rescuing" Proviral Sequences

The complementing viruses described above have many uses. One such use is to "rescue" unknown integrated viral genomes from cells. An unknown viral genome is "rescued" when the complementing virus provides packaging components in *trans* that recognize and package the unknown genome into a virus particle. The complementing virus have the potential ability to rescue some or all of the genomes of other viruses, *e.g.*, lentiviruses, oncornaviruses, papilloma viruses, adenoviruses, and alphaviruses. Such unknown viruses may be capable of inducing disease. Thus, complementing viruses are useful in diagnostic or exploratory procedures to rescue and to identify disease-causing viral agents in tissues. Furthermore, it may be possible to identify new viruses that are transmissible among humans for use as vectors for gene therapy.

No human retroviruses of the genus *Oncornavirus* have been identified. However, there have been reports of type C retrovirus particles in micrographs of human milk and the possibility exists that human disease could result from the infectious transmission of *Oncornaviridae* retroviruses (Wilkinson *et al., supra*). *Lentiviruses* are known to infect

humans. Rescue of retrovirus genomes with the complementing viruses of the invention thus has a diagnostic application.

Ex vivo Gene Therapy methods

Ex vivo methods for gene therapy involve transducing the cell *ex vivo* with a virus of this invention, and introducing the cell into the organism. The cells can be hematopoietic stem cells isolated from bone marrow or other cells that are in the host range of the packaged retrovirus particles of the invention. T cells are used in some embodiments in *ex vivo* procedures.

The same targeting strategy described above can be applied to the *ex vivo* transduction of cells, followed by re-infusion into a patient. For example, human umbilical vein endothelial cells can be transduced with a proviral sequence, followed by infection with a complementing virus of the invention. These cells are then used as feeder cells supporting the growth of CD34 positive stem cells. The umbilical vein cells are also used as producer cells that provide replication defective virus to the target CD34 cells. The CD34 positive stem cells would be cultured in the presence of a steady supply of replication defective virus, prior to reintroduction into a patient. This strategy can be applied by one of skill in the art to any suitable tissue.

Administration Replication Defective Virus

Replication defective viruses (for *in vivo* gene therapy) and transduced and infected producer host cells (for *ex vivo* gene therapy) can be administered directly to a patient, preferably a human. Administration is by any of the routes normally used for introducing a molecule or cell into ultimate contact with blood or tissue cells. The viruses of the invention are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such viral vectors in the context of the present invention to a patient are known to those skilled in the art.

Pharmaceutically acceptable excipients are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention. Formulations suitable for parenteral administration, such as, for example, by intra-articular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and

solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Parenteral administration and intravenous administration are suitable methods of administration.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular heterologous gene in the replication defective virus and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular virus or transduced cell type in a particular patient.

For administration, viruses and transduced cells of the present invention can be administered at a rate determined by the transduced cell type, and the side-effects of the virus or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses. For a typical 70 kg patient, a dose equivalent to approximately .1 μg to 10 mg are administered. Producer cells generating replication defective virus are optionally prepared for reinfusion according to established methods; see, *e.g.*, Abrahamsen (1991) *J. Clin. Apheresis* 6:48-53; Carter (1988) *J. Clin. Apheresis* 4:113-117; Aebersold (1988) *J. Immunol. Methods* 112:1-7.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Example: Production of a Replication Deficient Viral Vector

The following study demonstrates that the cell system and methods of the invention can be used to generate surprisingly high titers of replication defective viral particles containing a heterologous gene of interest by infection of stable cell lines into which proviral sequences have been stably integrated. As discussed above, the method of the invention incorporates a novel strategy which allows generation of very high replication defective viral particle titers in a system readily adaptable to scaled up production. This study also demonstrates that the method of the invention can be used to "rescue" retroviral genomes of known or unknown species.

A model system was constructed for this study by constructing several cell lines with integrated proviral sequences containing a model "heterologous gene of interest." The proviral sequence was designed to include an easily identifiable heterologous gene, specifically, the coding sequence for the green fluorescence protein (GFP) gene. The GFP gene was linked to the viral packaging signal from Moloney Murine leukemia virus (MMLV) Type C oncornavirus (see, *e.g.*, De Tapia (1998) *Biochemistry* 37:6077-6085; Fisher (1998) *Virology* 244:133-145; Mougel (1997) *J. Virol.* 71:8061-8065). The resulting GFP-LTR construct was then flanked by 5' LTRs from MMLV Type C retroviral vector (see, *e.g.*, Diaz (1998) *J. Virol.* 72:789-795; Faller (1997) *J. Cell Physiol.* 172:240-252).

The defective retroviral vector was packaged in PA317 amphotrophic retroviral env packaging cells (see, *e.g.*, Tortora (1994) *Cell Growth Differ.* 5:753-759; Sayers (1998) *J. Mol. Neurosci.* 10:143-16. The resultant PA317 "producer" cells (PA317/LGFP.neo producer cells) were cultured and the resultant supernatant was used for transduction (in particular, to transduce either D54 or A375 cells, see below).

The final construct was used to transduce and stably integrate into the DNA of several cell lines. In particular, human D54/GFP.neo glioma cells and A375/STK.neo cells were transduced. D54 and/or A375 cells were incubated with retroviral supernatant from

the PA317/LGFP.neo producer cells at an unknown MOI (not important because there is a selectable marker) in the presence of DMEM with 10% FCS and 8 ug polybrene/ml overnight. The cells were then selected in the presence of 1.1 mg/ml of G418 for 14 days. Integration and expression of the GFP segment was confirmed by FACS and by visualization using a inverted fluorescence microscope. STK expression (in A375 model) was tested by sensitivity of transduced cells to ganciclovir. This generated new cell lines with stably integrated proviral genomes. These transduced cell lines were used as the "producer cells" for infection (transduction) with a "complementing virus." The transduced D54 and A375 cell lines have been stable for at least 8 months.

A "complementing" viral construct was constructed by inserting various "complementing genes" into an adenovirus vector (vector described by, *e.g.*, Yoshida (1997) *Biochem. Biophys. Res. Commun.* 232:379-82). Specifically, genes encoding all essential *trans*-complementing functions necessary to "rescue" the integrated proviral sequence (described above) (*i.e.*, to allow the producer cells to generate provirus-containing replication defective viral particles), were inserted in the adenovirus. Various viral and retroviral genes were used as complementing genes. Specifically, the adenovirus was designed to express Moloney murine leukemia virus gag-pol protein ("MLV gag-pol" or "Axtet.gag-pol") (see, *e.g.*, Opstelten (1998) *J. Virol.* 72:6537-6545; Odawara (1998) *J. Virol.* 72:5414-5424; Odawara (1991) *J. Virol.* 65:6376-6379); vesicular stomatitis virus G envelope protein ("VSV-G env" or "Axtet.VSV-G") (see, *e.g.*, Abe (1998) *J. Virol.* 72:6356-6361; Liu (1996) *J. Virol.* 70:2497-2502); and, AVC3.rtTA, encoding a transcription factor (described by, *e.g.*, Gossen (1995) *Science* 268:766-9) which massively upregulates expression of Axtet.gag-pol and Axtet.VSV-G. The vesicular stomatitis envelope G-protein encoding gene was inserted to facilitate the infectivity of the resulting replication defective particle.

10^5 provirally transduced A375/STK.neo cells were infected (transduced) with this recombinant adenovirus. A375/STK.neo cells produce no replication defective viral vector in the absence of appropriate *trans*-complementing genes. However, when infected at multiplicities of 10 to 30 adenovirus plaque forming units (PFU)/ cell/ virus, replication defective vector production reached about 1000 pfu/cell/day. This compares very favorably with materials produced in transient systems (see, *e.g.*, Kinsella (1996) *supra*) and significantly outperforms stable cell lines (see, *e.g.*, Cosset (1995) *J. Virol.* 69:7430-7436). The cells had been transduced by incubation of adenoviruses at the MOI of 10 to 30

adenovirus plaque forming units (PFU)/ cell/ virus for 24 hrs in DMEM with 10% FCS. Media was then discarded. Cells were rinsed once with PBS, and then incubated in DMEM with 10% FCS.

Provirally transduced human glioma D54 cells were also infected (transduced) using a similar protocol. 10^5 provirally transduced human D54/GFP.neo glioma cells with this adenovirus vector. Supernatants were harvested at 48 hrs post-infection. 2.5 ml of supernatant harvested. Replication defective viral particle yield was titered on a human sarcoma cell line, TE671 cells. Titers were 10^9 pfu/ml, indicating that cells were producing 2,000 to 10,000 pfu/cell/day. Again, this result is far beyond that expected from the use of any described transient system or stable cell line-based system.

What is Claimed Is:

1. A method of producing a replication deficient viral vector encoding a heterologous nucleic acid, said method comprising the following steps:

i. transforming a producer cell with an integrating proviral sequence

wherein the integrating proviral sequence comprises a retroviral packaging signal and a sequence encoding the heterologous nucleic acid

wherein the proviral sequence is flanked by retroviral long terminal repeats;

ii. infecting the transformed producer cell with a complementing viral vector comprising a complementing genetic sequence

wherein the complementing genetic sequence comprises a gene which complements the integrating proviral sequence thereby enabling the producer cell to generate the replication deficient viral vector; and,

iii. culturing the transformed and infected producer cells in a culture medium under conditions permissive for the production of the replication deficient viral vectors.

2. The method of claim 1, where the method further comprises the isolation of the replication deficient viral vectors from the producer cell and the culture medium.

3. The method of claim 1, where the heterologous nucleic acid encodes a heterologous protein.

4. The method of claim 3, where the heterologous protein is a biologically active protein.

5. The method of claim 4, where the biologically active protein is selected from the group consisting of a hormone, enzyme, cell receptor, growth inhibitor, antiangiogenic factor.

6. The method of claim 5, where the biologically active protein is Factor IX, ADA or CFTR.

7. The method of claim 1, where the heterologous nucleic acid encodes an antisense nucleic acid or a ribozyme.

8. The method of claim 1, where the complementing viral vector is a vesicular stomatitis virus G.

9. The method of claim 1, where the complementing viral vector is an adenovirus.

10. The method of claim 1, where the complementing gene is selected from the group consisting of gag, pol and env genes.

11. A cell system for producing a replication deficient viral vector encoding a heterologous nucleic acid, said cell system comprising a producer cell, said producer cell having the following properties:

(a) having an integrated proviral sequence

wherein the integrated proviral sequence comprises a retroviral packaging signal and a sequence encoding the heterologous nucleic acid

wherein the integrated proviral sequence is flanked by retroviral long terminal repeats; and,

(b) having a complementing viral vector comprising a complementing genetic sequence

wherein the complementing genetic sequence comprises a gene which complements the integrated proviral sequence thereby enabling the producer cell to generate the replication deficient viral vector.

12. The cell system of claim 11, where the cell system further comprises means to isolate the replication deficient viral vectors from the producer cell and the culture medium.

13. The cell system of claim 11, where the heterologous nucleic acid encodes a heterologous protein.

14. The cell system of claim 13, where the heterologous protein is a biologically active protein.

15. The cell system of claim 14, where the biologically active protein is selected from the group consisting of a hormone, enzyme, cell receptor, growth inhibitor, antiangiogenic factor.

16. The cell system of claim 15, where the biologically active protein is Factor IX, ADA or CFTR.

17. The cell system of claim 11, where the heterologous nucleic acid encodes an antisense nucleic acid or a ribozyme.

18. The cell system of claim 11, where the complementing viral vector is a vesicular stomatitis virus G.

19. The cell system of claim 11, where the complementing viral vector is an adenovirus.

20. The cell system of claim 11, where the complementing gene is selected from the group consisting of gag, pol and env genes.

21. A kit for producing a replication deficient viral vector encoding a heterologous nucleic acid, said kit comprising:

(i) a first container containing a producer cell having an integrated proviral sequence

wherein the proviral sequence comprises a retroviral packaging signal and a sequence encoding the heterologous nucleic acid

wherein the proviral sequence is flanked by retroviral long terminal repeats; and,

(ii) a second container containing a complementing viral vector comprising a complementing genetic sequence

wherein the complementing genetic sequence comprises a gene which complements the integrated proviral sequence thereby enabling the producer cell to generate the replication deficient viral vector.

22. The kit of claim 21, where the kit further comprises means to isolate the replication deficient viral vectors from the producer cell and the culture medium.

23. The kit of claim 21, where the heterologous nucleic acid encodes a heterologous protein.

24. The kit of claim 23, where the heterologous protein is a biologically active protein.

25. The kit of claim 24, where the biologically active protein is selected from the group consisting of a hormone, enzyme, cell receptor, growth inhibitor, antiangiogenic factor.

26. The kit of claim 25, where the biologically active protein is Factor IX, ADA or CFTR.

27. The kit of claim 21, where the heterologous nucleic acid encodes an antisense nucleic acid or a ribozyme.

28. The kit of claim 21, where the complementing viral vector is a vesicular stomatitis virus G.

29. The kit of claim 21, where the complementing viral vector is an adenovirus.

30. The kit of claim 21, where the complementing gene is selected from the group consisting of gag, pol and env genes.

31. The kit of claim 21, wherein the first and second containers are combined.

32. The kit of claim 21, further comprising an instructional material, wherein the instructional material indicates how to use the producer cell and the viral vector to produce a replication deficient viral vector.

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 99/21393

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 C12N5/10 C07K14/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIN X. : "Construction of new retroviral producer cells from adenoviral and retroviral vectors." GENE THERAPY, vol. 5, no. 9, 7 September 1998 (1998-09-07), pages 1251-1258, XP000866154 the whole document ---	1-4, 9-14,19, 20
X	BILBAO G, ET AL: "Adenoviral/retroviral vector chimeras: a novel strategy to achieve high-efficiency stable transduction in vivo" FASEB JOURNAL FOR EXPERIMENTAL BIOLOGY, vol. 11, July 1997 (1997-07), XP000857999 BETHESDA, MD US the whole document --- -/--	1-4, 9-14,19, 20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 February 2000	09/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/US 99/21393

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 22143 A (UNIV ALABAMA AT BIRMINGHAM RES) 28 May 1998 (1998-05-28) the whole document ---	1-7,9-32
X	FENG, M. ET AL.: "Stable in vivo gene transduction via a novel adenoviral/retroviral chimeric vector" NATURE BIOTECHNOLOGY, vol. 15, September 1997 (1997-09), pages 866-870, XP000867001 the whole document ---	1-8
X	TOHRU ARAI ET AL.: "A new system for stringent, high-titer vesicular stomatitis virus G protein-pseudotypedA new system for stringent, high-titer vesicular stomatitis virus G protein-pseudotyped retrovirus vector induction by introduction of Cre recombinase into stable prepackaging cell lines." JOURNAL OF VIROLOGY., vol. 72, no. 2, February 1998 (1998-02), pages 1115-1121, XP000857996 THE AMERICAN SOCIETY FOR MICROBIOLOGY., US ISSN: 0022-538X figure 1 -----	1,11

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int.. ..nal Application No
PCT/US 99/21393

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WO 9822143 A	28-05-1998	AU 5447598 A	10-06-1998
		EP 0956052 A	17-11-1999